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One approach for the efficient intra-cellular delivery of proteins involves the use of protein transduction. We have demonstrated the feasibility of use PTDs for the treatment of cancer in murine tumor models, where a specific cationic peptide was able to transduce tumor cells efficiently following intra-tumoral injection. Intra-tumoral injection of a mitochondrial disruption peptide, KLAK, fused to a cationic transduction domain resulted in significant murine tumor apoptosis and complete tumor regression. Moreover, we have now shown that a peptide derived from the amino terminus of Smac, a protein able to block the anti-apoptotic effect of IAPs (Inhibitors of Apoptosis), induced apoptosis of DU145 and LNCaP cells following PTD-mediated internalization. Treatment with the PTD-Smac fusion increased the sensitivity of cells to apoptosis induced by TRAIL and etoposide treatment. In addition to the cationic, non-specific transduction peptides, we also have developed a method for screening for tissue-targeted transduction peptides using an M13 peptide phage display library. Initial screening of the library for transduction of tumors in vivo has identified peptides able to facilitate transduction of prostate tumors in vivo. These peptides will be developed for efficient delivery of therapeutic peptides and eventually proteins into prostate tumor cells for treatment of metastatic disease.

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Progress Report:

Introduction

Prostate cancer is the most common malignancy, excluding skin cancer, and the second leading cause of cancer death among men in the United States. Current therapies for prostate cancer include watchful waiting, hormonal therapy, targeted irradiation, and surgery. Although irradiation and surgery can cure prostate cancer if the cancer cells are confined, a significant percentage of the patients present with metastasis, particularly in bone. In addition, during the progression of disease, prostate tumor cells become hormone independent, making them unresponsive to hormonal therapy. Chemotherapy has not been used extensively to treat prostate cancer due to the low rate of proliferation. Since prostate tumors have certain changes in the activity or level of growth regulatory proteins, approaches to transfer the genes encoding these growth regulatory proteins have been tested. In particular, the use of vectors to deliver the genes encoding the regulators of cell growth, such as p53, p16 and Brca1, has been used clinically. However, even though the transfer of these genes to prostate tumor cells in culture results in reduced growth and even cell death, the low efficiency of delivery of the genes when administered in animal models and clinical trials has limited this approach. Thus alternative approaches for delivery of proteins able to stimulate death of prostate tumor cells or at least limit growth are needed. Recently, short sequences of amino acids, termed PTDs for protein transduction domains, have been shown to have the ability to mediate delivery of full-length proteins across cell membranes and into cell. This poorly understood process is called protein transduction and has been shown to be very efficient for delivery of proteins into cells in culture and in animal models. These short stretches of amino acids are able to deliver proteins, peptides, drugs, antibodies, and even nucleic acid into cells. Recently we have identified a class of peptides that are able to facilitate internalization of large protein complexes into mammalian cells including prostate tumors. Moreover, in preliminary experiments we have observed that certain transduction peptides are able to facilitate internalization of marker proteins into prostate tumor lines at least 50 fold more efficiently than previously reported transduction peptides. We also have demonstrated the ability to use these peptides for prostate cancer therapy by injecting a transduction peptide, fused to a tumor killing agent, into a murine tumor. Injection of the peptide resulted in complete tumor eradication. Thus transduction mediated by peptides could be used for efficient intracellular delivery of a wide spectrum of therapeutic agents. However, the transduction peptides identified to date are not prostate specific and thus can only targeted to prostrate tumors by the route of injection. To identify potential prostate-specific transduction peptides, we also have developed a method for screening for tissue-targeted transduction peptides. Using this method we have identified peptides able to transduce different cell types including one peptide able to transduce prostate tumor lines specifically. Thus the overall goal of the proposal is to develop approaches for optimizing and utilizing both general and targeted peptide transduction sequences for efficient delivery of anti-tumor agents into prostate tumor cells. The feasibility of treating prostate tumors in mouse models by transduction peptide mediated delivery of anti-tumor agents also will be evaluated. The proposed experiments will develop novel approaches for treating localized prostate tumors by direct injection of transduction peptides fused to anti-cancer agents as well as for the

treatment of metastatic disease by systemic administration of the transduction peptides. These peptide-based approaches for delivery of therapeutic agents would be of clinical utility.

Objective 1: To identify the optimal cationic transduction domain for prostate tumor cells as well as identify prostate tumor specific transduction domains.

Task 1. A panel of peptides, 4 to 12 amino acids in length and enriched for arginines and lysines will be screened for their ability to transduce three different prostate lines, DU145, LNCaP and PC3. The screening will be performed using biotinylated peptides coupled to two different marker complexes, avidin-ß-Gal and avidin-488. (Months 1-6, Year 1) .

Progress

We have screened panel of arginine and lysine rich transduction peptides for transduction of DU145, LNCaP and PC3 in cell culture. The most effective peptide for transduction was a peptide containing 8 lysines followed closely by peptides containing 8 or 6 arginines. Thus 8K and 6R will be used for all subsequent experiments.

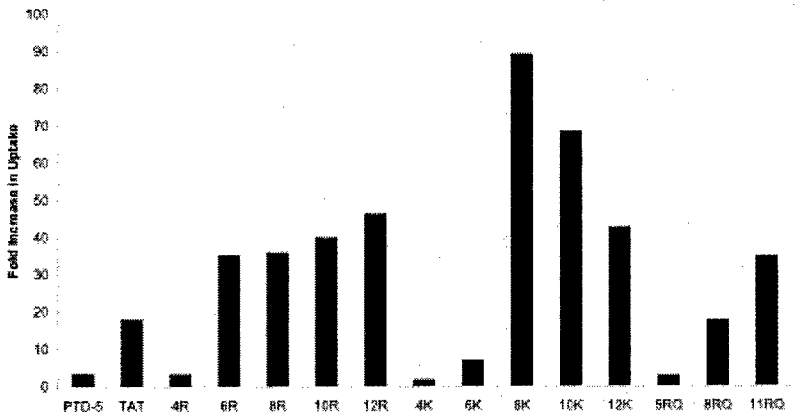


Figure 1: Summary of analysis of transduction by the indicated transduction domains. FITC labeled peptdides were examined for uptake into DU145 cells by flow cytometry.

Task 2. The four best peptides for transduction of prostate tumor cells will be characterized for intracellular localization by confocal microscopy following internalization by conjugation to avidin-488. The HIV Tat and PTD-5 peptides will be used as positive controls. (Months 3-9, Year 1)

Progress:

The 8K and 6R peptide are found to present in both the cytoplasm and nucleus of the cell, although the 8K appears to have more nuclear staining than 6R

Task 3. The optimal peptides (maximum of 2), based on their ability to deliver the ß-gal and 488 marker complex to the cytoplasmic and/or nucleus of the different prostate tumor lines in culture will be examined for ability to transduce prostate tumor cell *in vivo*. Nude mice will be inoculated subcutaneously with

LNCaP and DU145 cells. When the tumors reach palpable size, the peptide-avidin-ß-gal complexes will be injected intra-tumorally and the extent of transduction examined three hours post-injection by X-gal staining of tumor sections. The HIV and PTD-5 peptides will be used as a positive control. 3 mice will be used per treatment group. However, different doses of peptide-marker conjugates may have to be tested. (Months 6-12, Year 1)

Progress:

These experiments have been initiated.

Task 4. An M13 peptide phage display library will be used for screening for novel transduction peptides able to facilitate internalization in to prostate tumor lines in culture. The screen will be performed on DU145, PC3 and LNCaP cells with three rounds of screening. The phage that are isolated with be panned against HeLa cells to eliminate any non-specific internalizing peptides. (Months 1-12, Year 1)

Progress:

To screen for peptides able to facilitate tumor internalization, an M13 phage 12 amino acid control peptide display library (New England Biolabs, Beverly, MA) was used. Briefly, One nude mouse bearing a human tumor line was tail vein injected $1x10^{12}$ phage in 0.5 ml TBS, from M13 library for six hours. The mouse was sacrificed and the mouse lung, spleen, heart, kidney, liver, Brain and implanted glioma tumor were surgically isolated. The tissues were washed extensively (20 times) with pH 7.4 Tris-NaCl buffer, the cells were isolated by homogenization and trypsinization and lysed by three consecutive rounds of freeze thaw. The cell lysate were centrifuged and the supernatant removed and saved for phage titration and amplification. As shown in the below table, following the first round screen we recovered a number of phage from the tumor following freeze thaw, suggesting that the phage were internalized into the tumor following IV injection. We have performed a second round screen and are currently in round 3 of screening. As shown below, there is en enrichment of phage found internalized into tumor cells after three rounds of screening. Similar screens are being initiated in other prostate tumor lines. Overall, these results suggest that the approach to identify tumor specific transduction peptides is feasible.

Titration Results (first screen)

Titer	Plaques						
	Lung	**Liver**	**Spleen**	**Kidney**	**Heart**	**Brain**	**Tumor**
$1x10^1$	**U**	**U**	**U**	**U**	**U**	**U**	**U**
$1x10^2$	**U**	**U**	**U**	**U**	**U**	**U**	**U**
$1x10^3$	**184**	**U**	**U**	**U**	**490**	**890**	**U**
$1x10^4$	**9**	**U**	**U**	**U**	**42**	**37**	**406**

Third round of Tumor Biopanning by Tail Vein Injection of $2x10^{11}$ PFU of M13 Phage

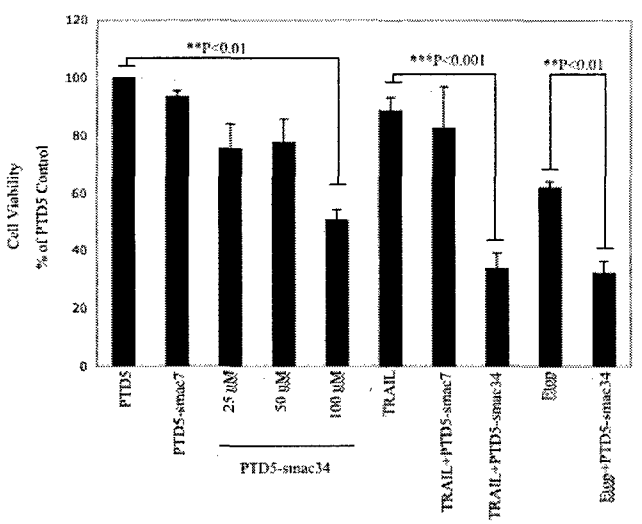
Titer	Plaques					
	Lung	Liver	Brain	Kidney	Heart	Tumor cells
$1x10^1$	U	U	U	U	U	U
$1x10^2$	U	U	U	U	U	U
$1x10^3$	U	U	206	U	604	U
$1x10^4$	509	U	34	210	96	U
$1x10^5$	84	960	2	16	3	1040

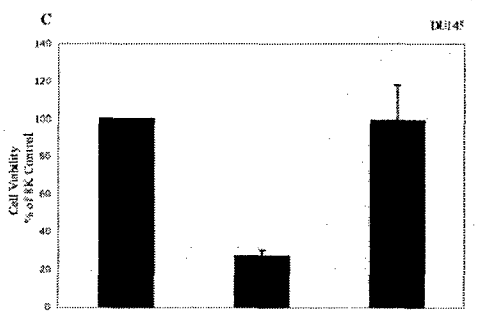
Objective 2: To examine the ability of peptide mediated transduction of specific agents to regulate prostate tumor cell growth and apoptosis.

Task 1. The ability of the 2 optimal cationic and the 2 optimal prostate targeted transduction peptides to deliver three different potentially therapeutic peptides will be tested. The three peptides to be tested include one derived from the amino terminus of Smac, a pro-apoptotic protein, a c-terminal p53 peptide able to activate the transcriptional activity of wildtype p53 as well as certain p53 mutants, and a general pro-apoptotic factor able to disrupt mitochondria. PTD-5 fused to the Smac, p53 and KLAK peptides will be used as positive controls. The peptide fusions will be examined for ability to inhibit viability or proliferation of prostate tumor cells in culture. Increasing concentrations of the different peptide fusions will be added to the media and cell viability measured at different time points by MTT and by FACS analysis following PI and annexin V staining. To examine the tissue specificity of the observed effects, the activity of the peptides will be compared in HeLa, MCF-7 and Saos-2 cells. (Months 1-12, Year 2)

Progress:

Prostate cancer cells eventually become resistant to apoptotic induction and are thus difficult to eradicate. Resistance results from mutations within the apoptotic pathway, such as the over-expression of the anti-apoptotic protein Bcl2. One way to circumvent this problem is to introduce pro-apoptotic proteins or peptides into prostate cancer cells using protein transduction technology. We choose to use peptides based on the amino terminus of mature Smac, a protein demonstrated to enhance





apoptosis by blocking IAP family members. The first set of peptides consisted of the protein transduction domain PTD5 linked to either the first 7 or first 34 amino acids of mature Smac via a diglycine bridge (PTD5-Smac 7, PTD5-Smac34 respectively). PTD5-Smac7 had no effect on the viability of the prostate cancer cell line DU145. In contrast, PTD5-Smac34 both enhanced TRAIL- and etoposide-mediated death, as well as induced cell death on its own. Cell death resulted from apoptosis as shown by Annexin-V staining and Western analysis of PARP cleavage (see Figure). In an effort to optimize the Smac peptides, a panel of protein transduction domains, (PTD), was tested for their ability to transduce DU145 cells. 8K proved to be the most efficient PTD for this task. Thus, the new peptides synthesized contained either the first 12 or 34 amino acids of mature Smac linked to 8K via a diglycine bridge, (Smac12-8K, Smac34-8K respectively). Smac34-8K efficiently induced and enhanced apoptosis whereas Smac12-8K remained ineffective (see figure). Smac34-8K also induced apoptosis in the prostate cancer cell line PP1C. Importantly, the induction of apoptosis by Smac34-8K is sequence specific as demonstrated by the inability of a scrambled Smac34-8K peptide to induce death. Lastly, studies using the pan-caspase inhibitor z-VAD-fmk illustrated that Smac34 induces apoptosis primarly through a caspase-independent pathway. Taken together, these experiments establish that peptides based on the first 34 amino acids of mature Smac may be a better therapeutic tool than shorter Smac peptides that only encompass the first 7-12 amino acids in the fight against prostate cancer.

A manuscript describing these results in close to submission (the above is the abstract from the paper) which will be provided to the DOD upon acceptance for publication.

Task 2. To ability of the p53-terminal peptides to induce endogenous p53 transcriptional activity will be examined by transfection of the prostate and non-prostate tumor cells with a p53-dependent luciferase reporter followed by addition of increasing concentrations of the p53 peptide fusions. The level of luciferase will be measured 6 hours post-addition of the peptide. (Months 1-6, Year 2).

Progress:

Work from our lab as well as work performed in the laboratories of several collaborators suggest that the effects of the c-terminal p53 peptide may not be sequence dependent, but instead may be based on a charge effect. This was done using a scrambled p53 c-terminal peptide that had a similar effect on cell viability as the c-terminal p53 peptide. Thus we are not pursing the p53 c-terminal peptide as a therapeutic agent.

Task 3. The ability of the p53 and Smac peptides to sensitize the tumor lines to the apoptosis effects of rTRAIL, etoposide and radiation will be examined. Increasing concentrations of the different peptide fusions will be added to cells followed by addition of suboptimal doses of rTRAIL and etoposide as well as radiation. (Months 6-12, Year 2; Months 1-6, Year 3)

Progress:

See task 1.

Conclusions:

We have screened panel of arginine and lysine rich transduction peptides for transduction of DU145, LNCaP and PC3 in cell culture, demonstrating that the most effective peptide for transduction was a peptide containing 8 lysines followed closely by peptides containing 8 or 6 arginines. These two peptides were found found to be present in both the cytoplasm and nucleus of the cell, although the 8K appears to have more nuclear staining than 6R. We also have used a peptide phage display library to screen for peptides able to target tumor cells following systemic delivery. In the first round screen, we recovered a number of phage from the tumor following freeze thaw, suggesting that the phage were internalized into the tumor following IV injection. These results suggest that the approach to identify tumor specific transduction peptides is feasible. Finally, in regard to therapeutic cargos, we have used peptides based on the amino terminus of mature Smac, a protein demonstrated to enhance apoptosis by blocking IAP family members. Smac34-8K efficiently induced and enhanced apoptosis whereas Smac12-8K remained ineffective. Smac34-8K also induced apoptosis in the prostate cancer cell line PP1C. Importantly, the induction of apoptosis by Smac34-8K is sequence specific as demonstrated by the inability of a scrambled Smac34-8K peptide to induce death. Lastly, studies using the pan-caspase inhibitor z-VAD-fmk illustrated that Smac34 induces apoptosis primarily through a caspase-independent pathway. Taken together, these experiments establish that peptides based on the first 34 amino acids of mature Smac may be a better therapeutic tool than shorter Smac peptides that only encompass the first 7-12 amino acids in the fight against prostate cancer.